Evolution of 2-long terminal repeat (2-LTR) episomal HIV-1 DNA in raltegravir-treated patients and in in vitro infected cells

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Objectives: Our aim was to analyse the evolution of HIV-1 2-long terminal repeat (2-LTR) circular DNA in vitro and ex vivo in the presence of raltegravir.

Patients and methods: Twenty-five patients starting a raltegravir-based regimen were included. Total HIV-1 DNA and 2-LTR DNA were quantified at baseline and in follow-up samples up to month 12. The effect of raltegravir on the formation of 2-LTR circles was evaluated in HeLa P4 cells. The effect of raltegravir was also investigated by sequence analysis of the 2-LTR circle junctions.

Results: Among 21 patients with undetectable 2-LTR DNA at baseline, 7 had detectable 2-LTR DNA during the follow-up. Three of four patients with detectable 2-LTR DNA at baseline had undetectable 2-LTR DNA during the follow-up (P=0.27). The mean 2-LTR level increased significantly (+0.07 log10/month, P=0.02) in raltegravir-treated patients, and a 2-LTR increase was also observed in raltegravir-treated HeLa P4 cells, with a peak at 3 days post-infection. 2-LTR DNA showed a high prevalence of deletions ex vivo (64.5%) and in vitro (50%) in the presence of raltegravir, which was not statistically different from the prevalence in untreated patients or cells.

Conclusions: In antiretroviral-experienced patients receiving raltegravir, 2-LTR DNA increased while total HIV-1 DNA decreased over time. The frequent rearrangements found in 2-LTR sequences warrant further investigations to determine the dynamics of evolution of unintegrated HIV-1 DNA.

Keywords: DNA circles, integrase, inhibitor

Introduction

The insertion of HIV-1 proviral DNA into the host cellular genome is catalysed by a viral enzyme, integrase (IN). IN catalyses two reactions: first, two nucleotides are removed from each 3’ end of the viral DNA (3’-end processing) and then the processed viral DNA ends are inserted into the host DNA (DNA strand transfer). IN inhibitors (INIs) target the viral IN, and several classes of INI targeting the viral enzyme and interfering with either the 3’-end processing step or strand transfer have been developed.1 INIs have been proved to block HIV replication in cell culture.1,2 Raltegravir (Isentress®, formerly known as MK-0518, Merck & Co.) is the first of this novel class of antiretroviral drugs and was approved by the US FDA in 2007 for treatment of HIV-1 infection. Raltegravir has shown potent antiretroviral activity in antiretroviral-experienced patients and in antiretroviral-naïve patients. Raltegravir antiviral activity is driven by IN strand transfer inhibition.3 A recent study investigated the effect of raltegravir and various IN mutants to raltegravir in HeLa P4 cells. The 50% inhibitory concentration (IC50; 10 nM) obtained for the wild-type virus confirmed the potency of raltegravir as an HIV-1 inhibitor.4 However, resistance mutations are present in the IN gene in a high percentage of patients who fail a raltegravir-based treatment.5 Several forms of unintegrated DNA are present in cells. The 2-long terminal repeat (LRT) DNA circle is generated by ligation of the cDNA ends by the host cell non-homologous DNA end-joining system. Recombination between the two LTRs of a linear DNA can yield 1-LTR DNA circles. Previous studies have shown that the presence of 2-LTR DNA circles increases under INI treatment in infected cells,1,6 although the mechanism underlying the increase is unknown. Svarovskaia et al.6 proposed that the viral DNA ends become more accessible to cellular nucleases and ligases when the integration reaction is inhibited.
Sequence analysis of the 2-LTR DNA circle junction showed the presence of deletions or insertions at the junction.

As shown in cell culture, inhibition of integration by INIs induces an increase in 2-LTR circles. To see whether the same situation occurs in vivo, we analysed the evolution of 2-LTR episomal HIV-1 DNA in 25 antiretroviral-experienced patients receiving a raltegravir-based regimen. In addition, we investigated the effect of raltegravir on the structure of the 2-LTR DNA circle junctions. Results were compared with the situation in raltegravir-treated cells.

Patients and methods

Patients and samples

The patients were selected from the ANRS Co3 Aquitaine Cohort, a prospective hospital-based cohort of HIV-1-infected patients in south-western France. Informed consent was obtained for all patients. The Aquitaine Cohort has an institutional review board (IRB) approval from Bordeaux University. Peripheral blood mononuclear cells (PBMCs) and plasma samples were collected at months 0, 1, 3, 6, 9 and 12.

Nucleic acid extraction

PBMCs were isolated from blood samples using Ficoll–Hypaque gradient centrifugation. Total DNA (including integrated HIV-1 DNA and episomal HIV-1 DNA) was extracted from patients’ PBMCs using the QIAmp blood DNA mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s protocol. HeLa P4 cells were infected with the HIV LAI strain. Viral DNA from infected HeLa P4 cells was isolated using the Roche procedure (High pure viral nucleic acid kit) and subjected to PCR.

2-LTR DNA circle and total HIV-1 DNA quantification

The 2-LTR DNA circles were amplified with primers HIV-F and HIV-R1 spanning the LTR–LTR junction, as described previously.7 Total HIV-1 DNA was amplified by quantitative real-time PCR using the light Cycler Instrument (Roche Diagnostics, Meylan, France) according to the manufacturer’s protocol. HeLa P4 cells were infected with the HIV LAI strain. Viral DNA from infected HeLa P4 cells was isolated using the Roche procedure (High pure viral nucleic acid kit) and subjected to PCR.

HIV-1 infectivity assay

Infectivity was tested on HeLa P4 cells expressing the CD4 receptor and carrying stably integrated β-galactosidase under the control of the HIV-1 LTR. 2-LTR DNA levels were measured every day up to 4 days post-infection. The reaction products were measured in a fluorescence micro-plate reader (Cytofluor II) at 405 nm.

Sequence analysis of 2-LTR circle junctions

DNA was extracted from patients’ PBMCs or infected HeLa P4 cells. Total cell DNA (1 μg) was used for 2-LTR DNA PCR amplification using the specific primers HIV-F and HIV-R1. 2-LTR DNA-containing PCR products were cloned and sequenced with M13 forward primer (ABI Prism big dye terminator cycle sequencing ready reaction kit, Applied Biosystems, Courtaboeuf, France).

Statistical analysis

Differences in HIV-1 RNA, CD4+ cell counts, total HIV-1 DNA and 2-LTR DNA were reported as difference from baseline to month 6 (M6) and M12. The frequencies and the nature of the circle junctions in both treated and untreated HeLa P4 cells and in both treated and untreated patients were compared using Fisher’s exact test. Repeated measurements of total HIV DNA and 2-LTR DNA were analysed using a linear mixed model with a random intercept (for total HIV DNA) and a random slope (for 2-LTR DNA) accounting for the intra-patient correlation. Distributions are described as medians (25th; 75th percentiles), unless stated otherwise. All statistical tests were two sided. Analyses were performed using SAS 9.1 (SAS Institute, Inc., Cary, NC, USA).

Results

Virological and immunological evolution in patients receiving a raltegravir-based regimen

The baseline characteristics of the 25 patients enrolled in this study are presented in Table S1 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. The evolution of plasma HIV-1 RNA copy number, CD4 count, total HIV-1 DNA and 2-LTR DNA copy number is depicted in Figure S1 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. A high proportion of patients had an HIV-1 RNA viral load <40 copies/mL at M6 and M12. We observed a median HIV-1 RNA decrease of −2.2 (−2.6; −0.1) log10 copies/mL at M6 and −2.5 (−2.9; −0.4) log10 copies/mL at M12. The median CD4 cell count increase was 36 (−14; 113) cells/mm3 at M6 and 48 (5; 111) cells/mm3 at M12. Virological failure (HIV-1 RNA >40 copies/mL) was observed in three patients [Figure S2 and Table S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)].

Evolution of 2-LTR episomal HIV-1 DNA in antiretroviral-experienced patients

The impact of raltegravir treatment on copy number of 2-LTR circles was assessed. 2-LTR DNA was detectable in 4/25 patients at baseline (patients B, D, L and U) and in 8/25 patients during the follow-up (patients C, D, F, M, Q, R, S and Y) (Figure S2). The 2-LTR DNA became undetectable in three of the four patients who had detectable 2-LTR at baseline. Among the 21 patients with undetectable 2-LTR DNA at baseline, in 7 it became detectable during the follow-up (P=0.27, McNemar test with Yates correction). Six of eight patients with detectable 2-LTR DNA during follow-up had undetectable plasma HIV-1 RNA during the same period (patients C, F, M, R and Y). The mean 2-LTR DNA increase from baseline to M6 and M12 was +0.14 ± 1.07 and +0.95 ± 1.37 log10 copies/10⁶ PBMCs, respectively. We observed a significant mean 2-LTR DNA increase of 0.07 log10 copies/10⁶ PBMCs/month during the follow-up (P=0.02).

The mean total HIV-1 DNA decrease from baseline to M6 and M12 was −0.21 ± 0.46 and −0.35 ± 0.26 log10 copies/10⁶ PBMCs, respectively. The mean total DNA decrease was −0.03 log10 copies/10⁶ PBMCs/month (P=0.001).

2-LTR DNA increased in the presence of raltegravir while total HIV-1 DNA decreased over time in raltegravir-treated patients. Of note, total HIV-1 DNA decreased between M0 and M6 in 7/8 patients with detectable 2-LTR DNA during the follow-up (median decrease: −0.30 ± 0.51 log10 copies/10⁶ PBMCs).
Kinetics of viral 2-LTR DNA in cells and sequence analysis of 2-LTR circle junction DNA ex vivo and in vitro

Raltegravir inhibited HIV infectivity in a dose-dependent manner in HeLa P4 cells (Figure 1a). The IC50 value (5 nM) confirmed the effect of raltegravir as an INI, which is in good agreement with previously reported results. The 2-LTR/total DNA ratio remained constant over 96 h post-infection without raltegravir (Figure 1b). In the presence of raltegravir, 2-LTR DNA increased, with a peak at 3 days post-infection, and decreased at 4 days post-infection. Quantification of the copy number of total viral DNA at 24, 48, 72 and 96 h post-infection showed a decrease in total HIV-1 DNA in the presence of raltegravir in infected cells (only 18% and 12% of the DNA detected at 24 h post-infection was still measurable at 48 and 96 h, respectively). The 2-LTR DNA/total HIV-1 DNA ratio indicated an increase in 2-LTR DNA circles with a peak 3 days post-infection and a decrease thereafter in the presence of raltegravir.

Sequence analysis of 2-LTR junctions was performed to see whether alterations in the sequence were observed in the presence of raltegravir (Table 1, and Figure S3 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]). The junction was frequently deleted in both raltegravir-treated and untreated patients in most of the PBMCs. 2-LTR DNA showed a high prevalence of deletions ex vivo (64.5%) and in vitro (50%) in the presence of raltegravir but this was not statistically different from the prevalence in untreated patients or untreated cells.

Discussion

This work is the first longitudinal study on 2-LTR circular HIV-1 DNA levels in PBMCs from patients with chronic HIV-1 infection receiving a raltegravir-based regimen. 2-LTR DNA became detectable during follow-up in 7/21 patients receiving a raltegravir-based regimen who had undetectable 2-LTR DNA at baseline, and in three of four patients with detectable 2-LTR DNA at baseline it became undetectable during follow-up. A significant increase of 2-LTR DNA during the follow-up in raltegravir-treated patients was observed. This increase remained significant after robustness analysis imputing either half of the detection limit for 2-LTR DNA or a value of 0 for measurements of ≤1 log10 copies/10⁶ PBMCs (data not shown). These results are in contrast to a recent study that observed no modification of total HIV DNA and 2-LTR DNA after a 24 week follow-up in patients with undetectable HIV-1 RNA switching to either raltegravir or enfuravir. The increase in 2-LTR DNA in some patients suggests that some pre-integration complexes (PICs) could still enter the nucleus of the host cells. The integration process is inhibited in the presence of raltegravir, and 2-LTR DNA circle formation increases. Whether the origin of PICs is due to an ongoing viral

![Figure 1. Kinetics of 2-LTR DNA in raltegravir-treated HeLa P4 infected cells. (a) Effect of raltegravir in HeLa P4 cells at 24 h post-infection. Successive dilutions of raltegravir were performed from 10 μM to 1 nM (gift from Merck & Co.). (b) Evolution of the 2-LTR/total HIV-1 DNA ratio in the presence of 10 μM raltegravir (filled squares) or without raltegravir (filled diamonds). The medium containing raltegravir (10 μM) was replaced every day to maintain the drug concentration. β-Galactosidase activity (%) was determined with respect to untreated cells. Data from a representative experiment performed three times are shown. Results given are means±SD.](image-url)
replication in sanctuary sites or a release of viral genomes from latently infected cells remains to be elucidated. Buzon et al.\textsuperscript{10} found a transient and significant increase in episomal HIV-1 DNA at week 2 compared with baseline in patients who were treated with a raltegravir-intensified regimen. Furthermore, the increase in 2-LTR circles was mainly observed in patients who intensified a protease inhibitor-containing regimen.\textsuperscript{10} We analysed the 2-LTR DNA kinetics in patients’ PBMCs up to 12 months. In raltegravir-treated HeLa P4 cells, 2-LTR DNA increased and reached a peak at 3 days post-infection before decreasing until 96 h. Results were similar to those observed when diketo-acid (DKA) is added to cells.\textsuperscript{6}

The analysis of sequences at the 2-LTR DNA circle junctions can provide insight into the metabolism of unintegrated viral DNA in the presence of INIs. Frequencies of wild-type junctions (52%), deletions (29%) and insertions (19%) of 2-LTR DNA circle junctions of untreated HeLa P4 cells were in agreement with previous studies.\textsuperscript{6} These DNA ends could be processed by host nucleases in order to be circularized in 2-LTR DNA circles. This is supported by a trend to a higher frequency of deletions in the presence of raltegravir.

In conclusion, we found an increase in 2-LTR DNA circles in the presence of raltegravir both in patients receiving a raltegravir-based regimen and in raltegravir-treated HeLa P4 cells. The exact nature of the molecular mechanism by which raltegravir leads to an increase in 2-LTR DNA circles remains to be elucidated.

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\section*{Transparency declarations}

None to declare.

\section*{Supplementary data}

Tables S1 and S2 and Figures S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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